Extracellular Microbial Synthesis of Gold Nanoparticles using Fungus *Hormoconis resinae*

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In this study, the fungus Hormoconis resinae *was screened from soil near a refinery and was found to produce stable gold nanoparticles extracellularly. The kinetics of the reaction was studied using UV–Vis spectroscopy and was further characterized by x-ray diffraction, energy dispersive x-ray (EDX) analysis, and high-resolution transmission electron microscopy. These analyses revealed that the gold nanoparticles are spherical and in nano-regime. The most important feature of* Hormoconis resinae *fungi is the following fact: they have a widespread presence in soil and can produce huge biomass. Such a cheap source of material gives the opportunity for cost-effective preparation of various gold-based nanostructures.*

INTRODUCTION

There is a growing need to develop environmentally benign nanoparticle synthesis processes that do not use toxic chemicals in the synthesis protocol. Even though many biotechnological applications such as remediation of toxic metals employ micro-organisms such as bacteria[1] and yeast,[2] such microorganisms were recently found as possible eco-friendly nanofactories.[3,4] Processes devised by nature for the synthesis of inorganic materials on nano- and micro-length scales have contributed to the development of a relatively new and largely unexplored area of research based on the use of microbes in the biosynthesis of nanomaterials.[5]

Studying nanomaterials biosynthesis offers a valuable contribution to materials chemistry. Many microorganisms can synthesize inorganic nanomaterial intra- or extracellularly[6] and they have received the most attention in the area of biosynthesis of nanoparticles. Early studies have revealed that microbes are able to reduce Au^{3+} ions to produce octahedral gold particles of nanoscale dimensions (5–25 nm) within bacterial cells by incubation of the cells with gold chloride[3,4,7] under ambient temperature and pressure conditions. Microorganisms such as bacteria, yeast, and now fungi play an important role in the remediation of toxic metals through the reduction of the metal ions that have been considered interesting as nanofactories very recently.[3,4]

How would you…

…describe the overall significance of this paper?

In this present work a novel method for the biological synthesis of gold nanoparticles is reported with the fungus Hormoconis resinae *which was isolated from soil near a refinery. Such a cheap source of material provides opportunity for cost-efficient synthesis of various valuable gold-based nanostructures.*

…describe this work to a materials science and engineering professional with no experience in your technical specialty?

In this synthesis method fungus Hormoconis resinae *was used, thus toxic chemicals and pathogenic substances are avoided during nanoparticles synthesis. These nanoparticles are extracellularly synthesized thus they are easy to extract from the solution and this would also contribute to cost-effectiveness of this method.*

…describe this work to a layperson?

In this work synthesis of gold nanoparticles has been reported by simple non-pathogenic fungus and these synthesized nanoparticles are in solution. Due to their resonance in visible regions they can be easily identified and no sophisticated expertise is required for their recovery.

Using the dissimilatory properties of fungi, the biosynthesis of inorganic nanomaterials using eukaryotic organisms, such as fungi, may be used to grow nanoparticles of gold[8] in *Verticillium sp.* fungal cells intracellularly.[9] It has been shown that fungi are excellent candidates in the synthesis of gold.[8,10] The fungus *Verticillium sp.,* when exposed to metal ions, releases enzymes that reduce the metal ions to yield highly stable nanoparticles in solution.[10,11] The extracellular production of metal nanoparticles by several strains of the fungus was described and demonstrated elsewhere.[12] The ability of some microorganisms such as bacteria and fungi to control the synthesis of metallic nanoparticles can be employed in the synthesis of new materials.[13] This extracellular enzyme shows excellent redox properties and it can act as an electron shuttle in the metal reduction. It was evident that electron shuttles or other reducing agents (e.g., hydroquinones) released by microorganisms are capable of reducing ions to nanoparticles. Jose-Yacaman and co-workers have demonstrated the synthesis of gold nanoparticles within live alfalfa plants by gold uptake from solid media.[14]

It has been reported that the Au^{3+} ions are reduced in the solid media to Au^0 by the plant and then the atoms are absorbed into the plant where the nucleation and growth of gold nanoparticles takes place. This method can be very efficient in decontaminating soil polluted with heavy metal ions.[14] Barring the fungus *Fusarium oxysporum*[10,15] and the actinomycete *Thermomonospora sp.,*[16] all other microorganisms and plants investigated thus far when challenged with metal ions have resulted in their reduction and growth of nanoparticles intra-cellularly. Such biosynthetic processes for the synthesis of nanoparticles would be more useful from the application point of view if the nanoparticles can be formed extracellularly. For the reasons mentioned above, monodisperse nanoparticles of gold were previously synthesized by using *Fusarium oxysporum*[17] and silver by *Hormoconis resinae.*[18]

A number of soil fungi have been screened for the efficient and rapid extracellular synthesis of metal nanoparticles and *Hormoconis resinae* (MTCC 368) has been identified as an exciting candidate for the synthesis of gold nanoparticles extracellularly.

See the sidebar for experimental procedures.

RESULTS AND DISCUSSION

In this work, fungus *Hormoconis resinae* was used for the synthesis of gold nanoparticles. Live biomass was prepared by inoculating pure culture in liquid growth medium at 26 ± 1°C, 150 rpm. Then biomass of *Hormoconis* was centrifuged at 5,000 rpm at 10°C for 20 min. and washed with deionized water then filtered. The carefully weighted 0.1 g biomass of *Hormoconis* was added to 50 mL of 1 mM aqueous $HAuCl_4$ solution, in a conical flask of 100 mL content at room temperature. The flasks were thereafter shaken at a rotation rate of 150 rpm at 30°C.

The biomass incubated with deionized water (positive control) retained its original color (i.e., yellow-green) while the gold chloride treated biomass turned purple (as shown in Figure 1) after 24 hours due to the formation of gold nanoparticles extracellularly. As indicated in the experimental section, the bioreduction was carried out at room temperature and clearly, the formation of gold nanoparticles was due to the fungal biomass.

The main groups of the enzyme secreted by biomass that may play an important role in reducing the $AuCl_4^-$ ions include the amino, sulfhydryl, and carboxylic groups[8,10,21] and the $AuCl_4^-$ ions could bind to biomass through these functional groups. The possible role involves the electron shuttle enzymatic metal reduction process. Previous studies[10,11,22] have indicated that NADH and NADH-dependent enzymes are important factors in the biosynthesis of metal nanoparticles. This fungus is known to secrete cofactor NADH and NADH-dependent enzymes that may be responsible for the bioreduction of $Au^{(3+)}$ to $Au^{(0)}$ and the subsequent formation of gold nanoparticles. The reduction seems to be initiated by electron transfer from the NADH by NADH-dependent reductase as electron carrier. Then the gold ions obtain electrons and are reduced to Au^0.[23] The exact mechanism of the reduction of gold ions is yet to be elucidated.

Reduction of the aqueous $AuCl_4^-$ ions during exposure to the fungus *Hormoconis* may easily be followed by UV–Vis spectroscopy. It is well known that gold nanoparticles exhibit an impressive purple color in water; this color arises due to excitation of surface plasmon vibrations in the metal

EXPERIMENTAL PROCEDURES

Fungal strain was isolated from soil samples, collected near a refinery situated at Mathura. Enumeration of the microbes present in the soil was done by serial dilution-agar plating method.[19] Serial dilution of soil suspension was prepared up to 10^{-6} dilution. 1 mL of suspension from dilutions 10^{-3} to 10^{-6} was transferred to the petri dishes containing Czapek's–Dox (CD) agar medium at 23±2°C for 6–8 days and growth was observed after two days. The experiment was set up in triplicate to minimize the deviations.

The fungi isolated on culture medium from soil were purified by spore suspension and streak method. The cultures were routinely (every 6–8 days) transferred onto fresh CD agar plates by streaking. Before fungal cultures were used for inoculation of liquid growth medium, the fungus was subjected to three transfers on CD agar plates by the direct agar transfer method.[20] The culture strains were identified as *Hormoconis resinae* (MTCC 368) IMTECH, Chandigarh. The pure cultures were maintained on czapek's-dox agar medium. The cultures were sealed by wax and kept at 4±1°C temperature in refrigerator.

The fungal biomass of *Hormoconis resinae* (MTCC 368) was cultivated in liquid growth medium using the shake flask method in a shaker cum incubator. Spores and mycelium from the CD spread plate cultures were transferred to 250 mL Erlenmeyer flasks containing 100 mL growth medium. Flasks were incubated at room temperature in a shaker cum incubator at 26 ± 1°C, 150 rpm for 5 days.

The gold chloride ($HAuCl_4$) used in the study was procured from Hi-media Company. In a typical synthesis of gold nanoparticles using *Hormoconis resinae* biomass, the carefully weighted 0.1 g biomass was added to 50 mL of 1 mM aqueous $HAuCl_4$ solution, in a conical flask of 100 mL content at room temperature. The flasks were thereafter shaken at a rotation rate of 150 rpm at 30°C. These synthesized nanoparticles were extracted from the solution by simple centrifugation at 12,000 rpm for 15 min. by REMI Ultra-microcentrifuge.

The bioreduction of Au^+ in aqueous solution was monitored by periodic sampling of aliquots (0.2 mL) of the suspension and then diluting the samples with 2 mL deionized water. Afterwards the UV–Vis spectra of resulting solution have been recorded. The UV–Vis spectroscopy analysis of synthesized gold nanoparticles was carried out as a function of bioreduction time at room temperature by ELICO UV spectrophotometer (Model No. BL-198).

X-ray diffraction (XRD) measurements of the *Hormoconis resinae* reduced gold nanoparticles were carried out on drop-coated films of the respective solutions onto glass substrates by a Phillips PW 1830 instrument operating at a voltage of 40 kV with Cu $K\alpha$ radiation.

The EDX was carried out with a Zeiss EVO 40 Electron Microscope with Bruker EDX. The EDX samples of the aqueous suspension of gold nanoparticles were fabricated by drop coating method. The EDX analysis was carried out in the spot profile mode with a beam diameter of 1 μm at several places on the sample. The results presented in this study are reproducible with the accuracy of ± 5% error.

Samples for high-resolution transmission electron microscopy analysis were prepared on carbon-coated copper grids. The film on the copper grids was allowed to stand for 2 min. and this process was followed with the removal of extra solution through blotting paper. Afterwards the grid was allowed to dry prior to measurement. The analysis was carried out by Tecnai G^2 F30 S-Twin (FEI; Super Twin lens with Cs =1.2 mm) instrument operated at an accelerating voltage of 300 kV (field emission gun) having a point resolution of 0.2 nm and lattice resolution of 0.14 nm.

nanoparticles.[24]

The surface plasmon resonance (SPR) band for gold nanoparticles occurs in the range 510–560 nm respectively in an aqueous medium. It is observed in Figure 2 that the gold surface plasmon resonance band occurs initially at 537 nm after 24 h and subsequently shifts to 541 nm as a function of time of reaction. After completion of the reaction, the wavelength of the surface plasmon band stabilizes at 541 nm. The absorption band for the gold nanoparticles solution is broad and slightly asymmetrical with indications of an additional weaker component at 630 nm. The appearance of this feature indicates either formation of stable aggregates of the gold nanoparticles in solution or shape anisotropy in the particles. Additionally, it is also observed that a weak absorption band above 630 nm also appears during the later stage of reduction of gold ions to form gold nanoparticles. The long wavelength band is observed to extend well into the near infrared region of the electromagnetic spectrum. This is attributed to the significant anisotropy in the shape of the gold nanoparticles with excitation of the in-plane SPR contributing to this feature or to the aggregation of particles in solution[25–28] and is attributed to excitation of the in-plane SPR. After completion of the reaction, the gold nanoparticle solution was separated from the fungal biomass and tested for stability. It was observed that the nanoparticle solution was stable for more than six months, with a little sign of aggregation. Because the proteins were secreted by the biomass,[9,29] the particles were stabilized in solution by a capping agent.

In Figure 3, the particles were confirmed as elemental Au^0 using x-ray diffraction (XRD). A couple of Bragg's reflections are distinctly exhibited, which may be indexed on the basis of the face-centered hexagonal structure of gold. The XRD spectrum shows four intense peaks, which agreed with the Bragg's reflection of gold nanoparticles as reported in the literature.[30] In earlier studies, the biological methods of synthesis gold nanoparticles[14,31] have been successfully carried out and substantiate our present finding of biosynthesis of gold nanoparticles.

The high-resolution transmission electron microscopy (HR-TEM) images illustrated the formation of gold nanoparticles by exposing 0.1g of *Hormoconis* biomass to 50 mL of 1 mM aqueous gold chloride solution at 26°C. The HR-TEM images recorded from drop-coated films of the gold nanoparticles synthesized by treating gold chloride solution with *Hormoconis* for 24 h are shown in Figure 4. A large density of spherical gold nanoparticles can be seen and the nanoparticles are quite ranged in size from 3–20 nm. Some hexagonal shaped particles are also present. Interestingly, particles are quite separated; this may be due to some bioorganic component secreted by the fungus acting as a stabilizing agent for the nanoparticles.

Figure 5 shows the energy dispersive x-ray spectroscopy (EDX) spectrum recorded in the spot-profile mode from one of the densely populated gold nanoparticles. Strong signals from the gold atoms in the nanoparticles are observed, while weaker signals from C, O, and Si atoms were also recorded. The C and O signals are likely to be due to x-ray emission from carbohydrates/proteins/enzymes present in the cell wall of the biomass while the Si signal is due to Si used as a substrate.

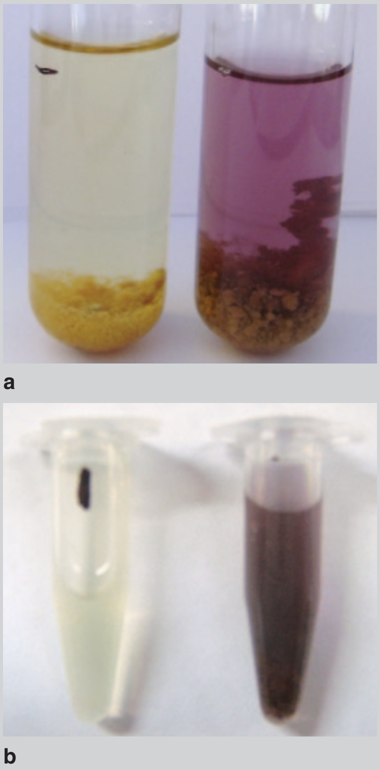

Figure 1. Tubes containing the *Hormoconis* biomass and biomass incubated with $HAuCl_4$ solution (a) after incubation of 24 h in an aqueous solution of $HAuCl_4$, (b) reference and gold nanoparticles after 6 months.

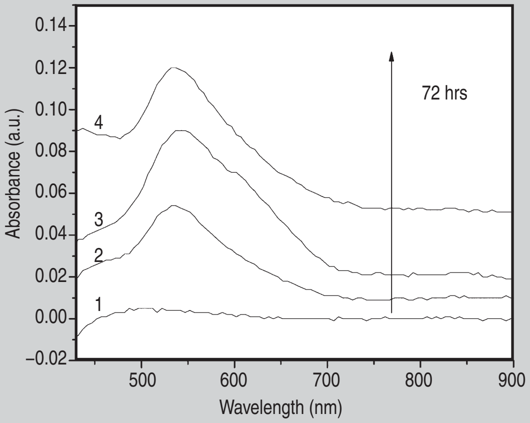

Figure 2. The UV-Vis spectra recorded as a function of reaction time of 1 mM $HAuCl_4$ aqueous solution treated with fungus *Hormoconis resinae*.

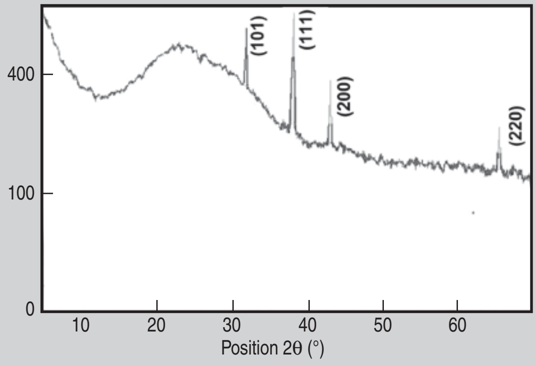

Figure 3. The XRD patterns recorded from drop-coated films of nanoparticles of gold synthesized using *Hormoconis resinae* on glass substrates. The Bragg reflections are indexed on the basis of the fcc structure.

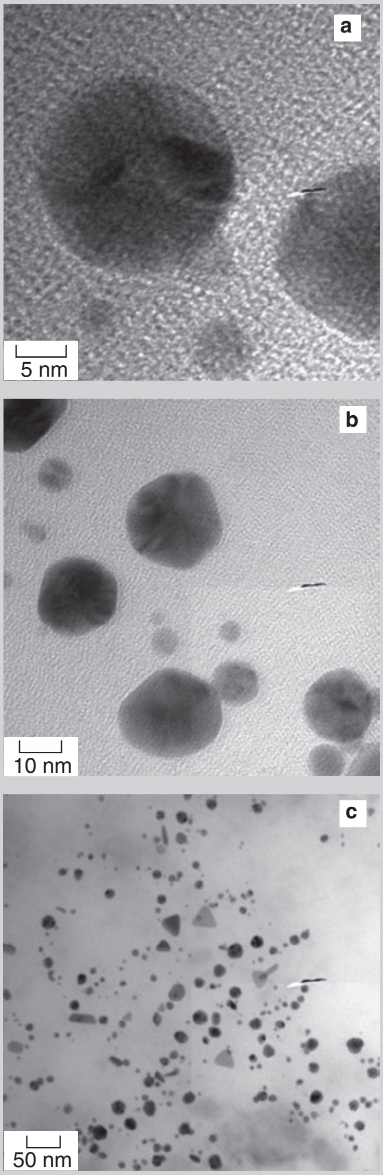

Figure 4. HR-TEM images illustrating the formation of gold nanoparticles by using fungus *Hormoconis resinae.*

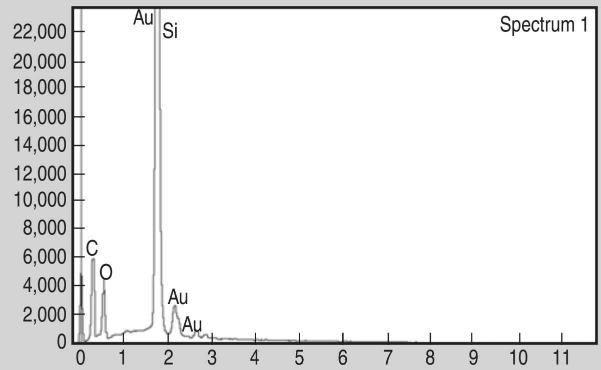

Figure 5. EDX of Au nanoparticles synthesized by treating 10^{-3} M $HAuCl_4$ aqueous solutions with fungus *Hormoconis resinae* and inset the particle targeted for EDX analysis.

CONCLUSION

Our results conclude that the fungus *Hormoconis resinae* is an excellent microbial resource for the extracellular synthesis of gold nanoparticles with appreciable stability in solution. The surface plasmon resonance band at 537 nm in UV-Vis spectrum indicates the primary evidence for synthesis of gold nanoparticles. The presence and size of gold nanoparticles in solution was further confirmed by the EDX and TEM techniques. The XRD spectrum shows that the gold nanoparticles were face-centered crystalline in nature. The sizes of these particles are measured with HR-TEM. To the best of our knowledge this is the first report showing extracellular synthesis of stable gold nanoparticles with *Hormoconis resinae*. The most important feature of *Hormoconis* fungi is the following fact: they have a widespread presence in soil near refinery and can produce a large amount of biomass. Such a cheap source of material gives the opportunity for cost-effective preparation of various gold-based nanostructures. Optimization of microbiological technique for synthesis of gold nanoparticles is yet to be worked upon.

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